Scanning Microscopy

Volume 2 | Number 1 Article 27

9-15-1987

The Bulk Specimen X-Ray Microanalysis of Freeze-Fractured, Freeze-Dried Tissues in Gerontological Research

I. Zs.-Nagy
University Medical School

Scanning Microscopy, Vol. 2, No. 1, 1988 (Pages 301-309) 0891-7035/88$3.00+.00
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

THE BULK SPECIMEN X-RAY MICROANALYSIS OF FREEZE-FRACTURED, FREEZE-DRIED TISSUES IN GERONTOLOGICAL RESEARCH

I. Zs.-Nagy

F. Verzár International Laboratory for Experimental Gerontology (VILEG), Hungarian Section, University Medical School, H-4012 Debrecen, Hungary

(Received for publication February 27, 1987, and in revised form September 15, 1987)

Abstract

The rationale for choosing the freeze-fracture freeze-drying (FFFD) method of biological bulk specimen preparation as well as the theoretical and practical problems of this method are treated. FFFD specimens are suitable for quantitative X-ray microanalysis of biologically relevant elements. Although the spatial resolution of this analytical technique is low, the application of properly selected bulk standard crystals as well as the measurement of the intracellular water and dry mass content by means of another method developed in the same laboratory, allow us to obtain useful information about the age-dependent changes of ionic composition in the main intracellular compartments. The paper summarizes the problems with regard to specimen preparation, beam penetration and the quantitative analysis of FFFD specimens. The method has been applied so far mainly for the analysis of intranuclear and intracytoplasmic concentrations of Na, Cl and K in various types of cells and has resulted in a significant contribution to our understanding of the cellular mechanisms of aging.

KEY WORDS: Bulk specimen X-ray microanalysis, freeze-fracture freeze-drying method, beam penetration in bulk specimens, standards for bulk specimen, quantitative microanalysis in bulk specimens.

Address for correspondence:
Imre Zs.-Nagy, DOTE, VILEG
H-4012 DEBRECEN, Hungary
Phone no. (36-52) 18-470.

Introduction

The basic principles and various features of the X-ray microanalytical method have been summarized in excellent books and review papers (Beaman and Isasi, 1972, Hall et al. 1974, Russ, 1974, Panessa, 1974, Barbi, 1979, Chandler, 1979, Newbury, 1979, Marshall, 1980, Roomans, 1979, 1980, Gupta and Hall, 1981, 1982, 1984; Hall and Gupta, 1983, 1984, 1986; Hall, 1986, etc.). Therefore, the aim of this paper is not to repeat the available information. The present paper is intended rather to summarize the available knowledge regarding a particular method of X-ray microanalysis specially developed in 1974-75 in our laboratory and having been used since that time to answer some defined questions of experimental gerontology. Although this method has been applied to quite a wide variety of problems (see for ref.: Zs.-Nagy 1983), its potential has remained underestimated by most authors. Therefore, it appeared desirable to summarize our experience in a tutorial way, in order to offer a comprehensive description for everyone who intends to apply this method in the future.

Why to choose bulk specimens?

The application of the X-ray microanalytical method to biological problems started during the late sixties when the electron probe X-ray microanalyzer and the conventional scanning electron microscope had been combined into a single instrument. There was a considerable enthusiasm among the biologists at that time, since the possibility of measuring elemental quantities as low as 10^{-18} g was offered by this method. To obtain at the same time also structural, distributional and localizational information about the particular element was very attractive. Unfortunately, however, this enthusiasm was not entirely justified for two main reasons explained below.

1. Although 10^{-18} g is a very small quantity, one has to recognize that at least this amount must be present in the analyzed microvolume, otherwise one cannot obtain a significant peak above the background intensity within a reasonably short time. However, most

of the elements of biological significance are present in the intracellular space in by far lower concentrations than would be necessary to achieve a spatial resolution obtainable in conventional electron microscopy of ultrathin sections. Therefore, when analyzing the concentration of natural components of the living material, one has to consider basically two possibilities: (i) in the case of thin specimens one has to increase the analyzed area laterally (and also in depth to a certain extent, as compared to the ultrathin sections), or (ii) in case of bulk specimens one is doing so in depth. Trace elements cannot even be mentioned as possible candidates for X-ray microanalytical work (Gupta and Hall, 1982).

2. The second disappointing fact was that even if one accepts great compromises with regard to the spatial resolution, it is still very troublesome to make suitable specimens in which all mobile, light elements remain at their original place. The best method known so far for a reliable preservation of the in situ elemental distribution in biological specimens is the quench-freezing which should be performed as quickly as possible. However, this must be followed by a proper method of exploration of the intracellular compartments to obtain a specimen suitable for X-ray microanalysis. We actually know two basic methods of such an exploration: (i) cryo-sectioning, and (ii) freeze-fracture. It is important to stress the general requirement that post-preparative diffusion artifacts must be avoided in these specimens.

The cryo-sectioning method of tissue exploration is quite a troublesome technique. It is outside the scope of the present tutorial paper to discuss the problems related to this methodology; sufficient information is available in numerous papers (Echlin, 1975, 1978, 1979, Somlyó et al. 1977, 1979a, 1979b, 1985, Ingram and Ingram, 1980, Hagler et al. 1980, Wendt-Gallitelli and Wolburg, 1981, Gupta and Hall, 1981, 1984, Moreton, 1981, Harvey, 1982, Roomans et al. 1982, Sitte, 1982, Zierold, 1982a, 1982b, 1983, 1986a, 1986b, Sumner, 1983, Hall, 1985, Meyer et al. 1985, Saubermann et al. 1981a, 1981b, etc). Although a considerable improvement has been achieved during the last decade in the methodology and application of the cryosection technique, it should be pointed out that during the early seventies when our laboratory started the application of X-ray microanalysis for gerontological research, both the frozen-hydrated or freeze-dried sections allowed us to recognize only the main cell and tissue compartments, like the nucleus, the cytoplasm, and the extracellular space (Dörge et al., 1974, 1978, Moreton et al. 1974). We have pointed out in our first relevant publication (Zs.-Nagy et al. 1977) that this level of structural resolution can easily be achieved even in freeze-fractured, freeze-dried (FFFD), non-coated, bulk specimens. Therefore, the author of this paper decided in 1974 not to follow the methodology of cryo-sectioning. The very first experimental results demonstrated that the bulk specimen analysis of FFFD biological tissues may deliver useful quantitative information, if proper analytical conditions are fulfilled. On the other hand, the preparation of such specimens is by far much quicker, easier and more reproducible than that of the cryo-sections. The validity of the bulk specimen approach has been accepted later by several authors (Marshall, 1980, Echlin et al. 1982, Echlin and Taylor, 1986).

Specimen preparation

One has to keep in mind that the in vivo distribution of the biologically occurring electrolytes can be maintained only by lowering the temperature of the living tissue as quickly as possible. This can be achieved, if small tissue pieces (about 1 x 1 x 2 mm) are excised from the (possibly living) animal and quenched in isopentane (or other cooling medium) cooled to its melting point by liquid nitrogen. In the case of isopentane this temperature is -160°C (Hodgman et al. 1959-60). The most practical method is just to drop the tissue piece in the isopentane in which solid and liquid parts are present. However, other methods of quench freezing may also be suitable.

The cells on the external surfaces of the tissue piece are obviously heavily damaged during the excision and are therefore not suitable for analytical purposes. Nevertheless, the cells localized inside the tissue piece remain practically intact, and one can explore these internal cells by fracturing the tissue piece while it is still in the frozen state. This can be achieved very simply by using a pair of scissors (precooled in isopentane): the tissue pieces must be broken across their longitudinal axis by the scissors, and due to the fact that the frozen tissue becomes hard and fragile, one obtains usually a quite nice breaking plane.

The freeze-fractured specimens must then be freeze-dried in order to remove the water. This can be done using various equipment. In our laboratory the vacuum evaporators (JEOL JEE-B or JEE-4X equipped with the EE-ACE cooling attachment) are used for this purpose. This system allows us to keep the specimen holder in a temperature range from -80 to -120°C, whereas the "cold finger" is kept at liquid nitrogen temperature. The minimum distance in this system between the specimen holder and the "cold finger" is 30 mm, and the highest vacuum is in the range of 10^{-5} - 10^{-6} Torr. At a specimen temperature between -80 to -100°C in this instrument (on average about -90°C), the tissue pieces dry without reaching the recrystallization temperature of ice (about -70°C), i.e., the original distribution of the light, mobile elements such as sodium and potassium is maintained. The vacuum and the temperature differences mentioned above should be kept in the system for about 4-5 h (we can place 6 types of tissue, 2-4 pieces each at one time in the specimen holder), sufficient for drying the tissue pieces. It should be noted that the parameters of our freeze-drying procedure have been established empirically, nevertheless, later theoretical work confirmed

their validity. Namely, the drying time per 1 mm ice was given by Umrath (1983) for this temperature interval as ranging from 3.6 h to almost 6 days if the pressure is in the range of 10^{-4} - 10^{-5} Torr. Considering that in our system the vacuum is at least an order of magnitude better and the specimen size is within 1 mm, the schedule of freeze-drying described above results in a suitable drying of the tissue pieces. The time required may also depend on the tissue quantity freeze-dried at the same time. After 5 h we suspend the cooling of the specimen holder. Subsequently, the temperature rises gradually to room temperature within about 2 h. The "cold finger" is cooled maximally even during this period. In order to be sure that no water-condensation takes place on the specimens when the vacuum is suspended, the temperature of the specimen holder is increased by the incorporated heating element to about 30-35°C, before the vacuum is broken. The specimens are removed and the "cold finger" is warmed up and dried. Afterwards the specimens are stored in vacuum in the same evaporator where they can be maintained at about 10^{-2} - 10^{-3} Torr practically without time limitation and risk for electrolyte redistribution.

Electron microscopy of the FFFD specimen

The FFFD specimens can be handled very easily under a light microscope. The freeze-fractured surface can be recognized with certainty since it is devoid of blood contamination, i.e., it appears almost completely white after freeze-drying, whereas the other cut surfaces of the tissue block always show some "dirty" appearance because of the rest of blood. The selected specimen(s) can be mounted easily on the regular specimen holders of any scanning microscope by simply attaching them by means of a conductive glue. In certain types of electron microscopes one might have to modify the specimen holder somewhat but this is not a serious problem.

Extensive evidence has been published regarding FFFD specimens of liver, brain, tumor, etc., tissues demonstrating that they can be investigated in the scanning electron microscope without any coating layer (Zs.-Nagy et al.1977, 1981b, 1983, Zs.-Nagy, 1983, Bertoni-Freddari et al. 1981, etc). Even as long as 1 min exposure times did not give blurred or out-of-focus micrographs, i.e., the surface charging can be neglected. The effective beam current in these specimens proved to be in the order of magnitude of 1-2 pA (Zs.-Nagy et al. 1982). The low charging may be due to the fact that the total bulk conductivity of the FFFD specimen is comparable to that of the carbon coatings. As a matter of fact, one never encounters very strong electrostatic charging of the specimen, if the accelerating voltage is kept in a reasonably low range (10-20 kV). Other authors (Marshall, 1980, Echlin et al. 1982, Echlin and Taylor, 1986) using bulk specimens cover the surface with various layers (carbon, Al,Cr). However, in this case the preparative work becomes more time-consuming, and the quantitative analysis should consider the contribution of coating layer to the background. On the the other hand, one gets a "nicer" morphology of the specimen surface.

It has been shown in various papers from our laboratory (Zs.-Nagy et al. 1977, 1981b, 1983, Bertoni-Freddari et al. 1981), that using the secondary electron image mode on the FFFD specimen one can recognize all the major tissue and cell compartments, like the extra- and intracellular space, nucleus, cytoplasm, various cell types, red blood cells, etc. X-ray microanalysis can be performed at any place on the upper surface either in point form, or in regular scanning mode according to one's choice. It is important, however, to consider the penetration depth of the electron beam in the given specimen, in order to obtain an estimation about the real size of the analyzed volume.

The problem of beam penetration

One can calculate values for the penetration depth of a given accelerating voltage from the physical theory using the so-called range-equations (Beaman and Isasi, 1972). In one of our previous papers (Zs.-Nagy et al. 1982) we have shown that these calculations may give considerably different values, since the constants used by various authors are different. Therefore, it appeared more appropriate to perform some direct measurements on this parameter (Zs.-Nagy et al., 1977), in order to be sure about the maximum depth of the origin of the collected X-ray photons from a given cellular area. These types of investigation which can be carried out for any type of tissue or material, may be useful. Therefore, they are summarized below.

The concept of the method used can be called the sandwich technique. Sectioned layers of Araldite (or tissue) of various thicknesses are layered onto a foil of aluminium and this sandwich is brought into the analytical electron microscope. One should position this specimen in the microscope so that the pure Al and the sandwich can be seen with very little movement of the specimen stage. The two parts are clearly recognized simply on the basis of their morphology. One can perform X-ray microanalysis on the pure Al surface as well as on the sandwich using standardized microscope parameters (accelerating voltage, nominal beam current, magnification, scanning speed, tilting, etc). Obviously, the highest intensity of the Al-peak is obtained on the pure Al surface, whereas the sandwiches will give lower Al-peaks with increasing thickness of the superposed layer. In this way one can determine the thickness of the section at which no Al X-rays reach the detector. This section thickness indicates in practice the maximum penetration depth reached by the electrons with sufficient energy to excite the Al below the section. More precisely, there is a situation in which the Al excitation and the absorption of the Al X-rays by the section are equal, i.e., no Al is detected. Similar studies

were performed in which copper instead of Al was used and the results can be found in detail in Zs.-Nagy et al. (1977). The main conclusions are that (i) this method gives results in the range calculated on the basis of a theoretical approach; (ii) in FFFD liver specimens the maximum penetration depth of 10 kV electrons is about 4-5 μm, i.e., when the analysis is performed at this accelerating voltage, the analyzed microvolume may remain within half of a hepatocyte nucleus; (iii) using this principle for various tissues one can always control the penetration depth of the exciting electron beam under standardized conditions. It is important to realize that the maximum penetration depth is somewhat different for various elements: e.g., for Na it is larger, whereas for K it is smaller than for Al. It should also be noted that the necessary overvoltage ratio for a satisfactory excitation of the elements to be analyzed is estimated to be about 2.0 - 2.5 (Russ, 1974). Using 10 kV electrons this requirement is satisfied in practice for all biologically occurring elements including the calcium.

Recording X-ray spectra in FFFD specimens

The surface of the FFFD specimen does not permit high spatial resolution in morphological terms. Nevertheless, one can select regions in the nucleus or cytoplasm for microanalysis. By increasing the magnification up to about x30,000 - 100,000 one can obtain quite a small analyzed area (although obviously the penetration depth has to be taken into account). At such a high magnification no structural details can be recognized. However, lowering the magnification again after the analysis had been performed, one can clearly recognize where the beam was positioned during the analysis, since one can see a slight contamination spot on that place. Usually a 40 second analysis time with a cps of 400 - 500 is quite sufficient to obtain a suitable spectrum in which one can recognize the most important peaks like sodium, magnesium, phosphorus, sulfur, chloride, potassium and (only in some tissues) calcium. The analysis of this latter element represents a great difficulty in biological materials, since we usually find a very high intracellular potassium content, and the K_β line of this element strongly overlaps with the K_α of calcium. In addition, the calcium concentration in the cytoplasm is usually much lower than that of potassium. Therefore, any separation method of the overlapping peaks is rather difficult. The handling of the obtained spectra depends on the available computer capacity of the system used. However, quantitative data could be obtained on the FFFD specimens even by means of the most primitive computer systems represented by the EDAX Micro Edit system (Zs.-Nagy et al. 1977), which was available long before the microprocessors.

Quantitative analysis

One of the greatest problems in the X-ray microanalysis is the proper selection of the method of quantification of the collected data. The most reliable method according to our experience for the FFFD specimens proved to be the mass-fraction method of Hall et al. (1973). This method had originally been elaborated for thin specimens, nevertheless, its validity for thick specimens has also been demonstrated (Millner and Cobet, 1972, 1973) and adopted later on also by others (Marshall, 1980, Echlin et al. 1982, Echlin and Taylor, 1986). The principle of the method is as follows:

$$C_x = A_x \frac{(n_x/n_w)_{sp}}{(n_x/n_w)_{st}} (N_x/\Sigma NZ^2)_{st} \overline{(Z^2/A)}_{sp} \quad (1)$$

where C_x is the mass fraction of element x, A is the atomic weight of element x or any other elements in the standard, n_x and n_w represent the counts in the peak and the background, respectively, sp and st indicate the specimen and the standard, N_x stands for the number of the atoms of element x in the standard molecule, whereas N indicates the number of the atoms of any element in the standard, Z is the respective atomic number.

Equation (1) can be used for quantitation, if proper standards can be found and measured. However, according to the physical theory, bulk samples require a correction for the interelement influences, called ZAF correction. Although the computer capacities available today could perform this type of correction without any problem, the validity of ZAF correction methods is doubtful for the light elements distributed in an organic matrix because of the unsatisfactory exactness of the physical constants, etc. (Russ, 1974). Therefore, one had to find a compromise allowing us to perform the quantitative analysis even without ZAF correction. We shall demonstrate here a simple but powerful method which has been elaborated for this purpose (Zs.-Nagy and Pieri, 1976, Zs.-Nagy et al. 1977).

Equation (1) implies that the value:

$$Y = (n_x/n_w)_{sp} \overline{(Z^2/A)}_{sp} \quad (2)$$

is directly proportional to the mass fraction of element x (C_x), since all other expressions of the equation (1) are constant for a given case. Equation (2) allows us to check whether bulk crystals of known composition can be used as standards without ZAF correction or not. Namely, if various standards are compared to each other, i.e., they are considered as specimens, the correlation between Y and C_x will tell us whether a ZAF correction is necessary or not. Using an empirical selection of standard bulk crystals containing potassium, sodium and chloride we have established that a good positive linear correlation (r is in the range of 0.9920 - 0.9997) exists between Y and C_x, if the crystals are chosen so that apart from the main element of interest, the atomic number of the other accompanying elements is lower than

Z = 11. In such cases, the accompanying elements were generally C, O, N, and H, i.e., the composition was, in principle, very similar to the biological matrix. For a number of crystals listed in our previous papers (Zs.-Nagy and Pieri, 1976, Zs.-Nagy et al. 1977) there were no technical difficulties when performing the analysis, however, some of them proved to be unsuitable. (For example, K-oxalate is very strongly decomposed by the electron beam, in agreement with the observations of others: De Meis et al. 1974). Using this possibility, we calculated a factor (F) from the equation (1) as follows:

$$F = A_x \frac{(N_x/\Sigma NZ^2)_{st}}{(n_x/n_w)_{st}} \overline{(Z^2/A)_{sp}} \qquad (3)$$

The value of F was calculated for all standard crystals for a given element of interest and averaged. The FFFD specimens display a value of the mean $(Z^2/A)_{sp} = 3.28$ (Hall et al. 1973); this figure was used in each case for our calculations (Zs.-Nagy et al. 1977). The only information we need from the X-ray spectrum for this type of analysis is the net peak integral (n_x) as well as the value of n_w, i.e., the "white" counts, or the background integral. Optimally n_w should be determined below the peak of element x, but since not all software allows the calculation of it, one can also take n_w from any other part of the spectrum, provided that no significant peak is present there and the same energy range is used also for the standards. A further requirement is that no excessive absorption of X-rays should occur in the specimen (and the standards) due to the problems of surface geometry such as a very uneven surface. Further information relevant to this problem can be found in the papers of Roomans (1981) and Wroblewski et al. (1983). In case of FFFD specimens the energy range of 4 - 6 keV proved to be suitable for this purpose. Therefore, in all our studies, the value of n_w was taken from this range. The value of F can be determined so that $F(n_x/n_w)_{sp}$ directly gives the mass fraction of element x in the specimen in %. It is important to stress here that values of F are valid for a given instrumental configuration. If parameters such as the specimen-detector distance, the take-off angle of the X-rays, the accelerating voltage, etc, are changed, new measurements must be performed on the standards to obtain the values of F valid for the new situation.

One can perform calculations to show that this method of avoiding the ZAF correction may introduce some systematic error in the final elemental concentrations, but due to the fact that the elements K, Na and Cl occur in the FFFD type specimen in rather low concentrations, this error is certainly negligible. On the other hand, the simplicity of this technique permits the collection of a great number of data within a relatively short time: one analysis requires 40 sec and together with the calculations and selection of the next cell to be measured, it can be performed within 1 min. Even if the cryosection techniques available today can permit a similar speed of getting data, they suffer from the disadvantage that cryosections of reproducible quality are required (Zierold, 1986b) for numerous purposes, which is a really difficult task even today.

The analytical results obtained on the FFFD specimens as described above obviously give mass fraction values for the dry mass of the intracellular compartments. If one intends to transform these data to wet concentrations, it is necessary to measure the intracellular water content. We have elaborated on an X-ray microanalytic method for such a purpose, which avoids the use of any standards (Lustyik and Zs.-Nagy, 1981, 1985, 1987, Zs.-Nagy et al. 1982). It can be successfully applied to freeze-fractured frozen-hydrated specimens in a scanning electron microscope which in addition to the X-ray microanalytical system, possesses a Cryo Unit suitable for the handling and micromanipulation of frozen-hydrated specimens; this method is described in detail in a parallel paper (Lustyik and Zs.-Nagy, 1987).

The mathematical form used to obtain the elemental concentrations in units of mEq/kg intracellular water is the following:

$$C_x = \frac{R_d F_x C_d 10^6}{(1 - C_d) A_x} \qquad (4)$$

where R_d is the peak to background ratio of the element x measured in the FFFD specimen; F_x is the factor derived from the standards for the element x as defined by Equation (3); C_d is the dry-mass fraction of the intracellular space, i.e., $(1 - C_d)$ is the water-mass fraction (both determined as described by Zs.-Nagy et al. 1982, Lustyik and Zs.-Nagy, 1981, 1985, 1987); and A_x is the atomic weight of the monovalent element x (or the equivalent weight, if element x is not monovalent). Suitable utility computer programs can be used for a straightforward calculation of the elemental concentrations, if the data requested are available.

A survey of the results obtained

Since the application of the FFFD bulk specimen method of X-ray microanalysis to experimental gerontology and cancer research has recently been reviewed (Zs.-Nagy 1983), the present paper does not describe detailed results. However, it seems to be necessary to list the most important data also here for three main reasons. (i) The reader of this paper can directly find references to judge the reliability of this method. (ii) An outline about the general biological background of these studies may be important for those who are interested. (iii) Some recent progress in the field of experimental gerontology can be illustrated.

The first observations using the FFFD bulk specimen method were made on age-depen-

dent changes of the intracellular monovalent electrolyte contents in rat brain and liver cells (Pieri et al. 1977). Although at that time we did not have a method for measuring the intracellular water and dry mass content, it was obvious even on the basis of a hypothesized age-dependent dehydration of the intracellular mass that aging causes a considerable increase of the monovalent ionic strength in the cytoplasm and nucleus of nerve cells and hepatocytes. These first results were later confirmed when the water content measurements had been performed (Lustyik and Zs.-Nagy, 1981, 1985, 1987, Zs.-Nagy et al. 1982). The development of the membrane hypothesis of aging (MHA) (Zs.-Nagy 1978, 1979, 1985, 1986, 1987a, 1987b, Zs.-Nagy and Semsei, 1984) was possible on the basis of the first X-ray microanalytic observations using the bulk specimen method.

The MHA represents a cell-physiological mechanism which can explain the aging process with general validity. The essential point of this hypothesis is that the general physicochemical background of the aging process is the cross-linking effect of the oxygen free radicals, especially of the $OH^{\cdot}$ radicals. The $OH^{\cdot}$ free radical reactions are basically density-dependent, therefore, they result in more intermolecular damage where the molecules are nearer to each other. Since the most compact structures of the cells are the cell membranes, the rate of damage there is higher than in the cytosolic components. The cell plasma membrane is exposed to additional damage caused by the so-called residual heat deriving from the action potentials (see for ref.: Zs.-Nagy 1979). As a consequence, the leading point of cellular aging is a gradual loss of permeability of the cell membrane for potassium (and probably for water), which, on the other hand, results in a gradual increase of the intracellular potassium concentration and overall density. It is well established that all enzyme activities show an inverse exponential correlation with the density of their molecular environment (see for ref.: Zs.-Nagy 1986). The increase of the intracellular ionic strength and density up to a certain level is a phenomenon inherent to cell maturation and differentiation. However, this process does not stop at a certain age. Therefore, the continuous "maturation" becomes a suicide process for the cells, since the increasing intracellular density will cause a decrease of all enzyme activities. Among other factors, enzymes participating in the direct radical defense (e.g., superoxide dismutase, catalase, glutathione peroxidase) become less efficient. Furthermore, the enzymes participating in the elimination and replacement of the damaged components will also be insufficient. The result of this situation is a dehydration of the body, an increase of the cross-linked proteins (Lustyik and Zs.-Nagy, 1985, Nagy and Zs.-Nagy, 1984, Zs.-Nagy et al. 1981a) as well as of the waste-products (lipofuscin) in the cells, accompanied by a slowing down of the RNA synthesis rate (and protein turnover) (Richardson and Semsei, 1987, Richardson et al. 1985, Semsei et al. 1982). These results clearly illustrate how important the application of the FFFD specimen method was to the experimental gerontological problems during the last 12 years.

Some recent results should also be mentioned. For example, the Rb^{+}-discrimination ratio has also been measured in the brain and confirmed the age-dependent increase of this parameter observed before on the liver (Gyenes et al., 1984). It has also been described that the Rb^{+}-discrimination ratio behaves quite similarly in vitamin-E deficient rats as during normal aging in the brain cells (Pieri et al. 1986).

A special application of the FFFD specimen X-ray microanalysis was elaborated for an indirect determination of the resting, passive permeability ratios for potassium and chloride in the snail Lymnaea stagnalis L., where intracellular microelectrophysiological measurements were first carried out on an identified giant neuron of the left parietal ganglion, and subsequently the intracellular monovalent ion as well as water concentrations were measured on the very same cells by using the bulk specimen X-ray microanalysis. Details can be found in our paper (Zs.-Nagy et al. 1985). The investigations on the intracellular monovalent electrolyte content have been extended also to heart and skeletal muscle cells (Lustyik, 1986, Von Zglinicki and Lustyik, 1986).

Conclusion

The main conclusion of this paper is that the FFFD bulk specimen X-ray microanalytic method allows rather easily a great number of data regarding the intracellular concentrations of the monovalent electrolytes to be obtained. Although the obtainable information in practice remains at the cellular level, i.e., no high spatial resolution is achieved, these data are of great importance for the understanding of the cellular mechanisms of the maturation and aging. This method can successfully be applied to other fields where the average intranuclear or intracytoplasmic elemental concentrations should be considered.

References

Barbi NC. (1979). Quantitative methods in biological X-ray microanalysis. Scanning Electron Microsc. 1979; II: 659-672.

Beaman DR, Isasi JA. (1972). Electron Beam Microanalysis. American Society of Testing Materials, Philadelphia, PA. pp.: 1-80.

Bertoni-Freddari C, Giuli C, Lustyik Gy, Zs.-Nagy I. (1981). In vivo effects of vitamin E deficiency on the intracellular monovalent electrolyte concentrations in brain and liver of rat. Mech. Ageing Dev. 16, 169-180.

Chandler JA. (1979). Principles of X-ray microanalysis in biology. Scanning Electron Microsc. 1979; II: 595-606.

De Meis L, Hasselbach W, Machado RD. (1974). Characterization of calcium oxalate and calcium phosphate deposits in sarcoplasmic re-

ticulum vesicles. J. Cell Biol. 62, 505-509.

Dörge A, Gehring K, Nagel W, Thurau K. (1974). Intracellular Na^+-K^+ concentration of frog skin at different rates of Na-transport. In: Hall T, Echlin P, Kaufman R. (eds.): Microprobe Analysis as Applied to Cells and Tissues. Academic Press, New York, pp.: 337-350.

Dörge A, Rick R, Gehring K, Thurau K. (1978). Preparation of freeze-dried cryosections for quantitative X-ray microanalysis in biological soft tissues. Pflügers Arch. 373, 85-97.

Echlin P. (1975). The preparation of frozen-hydrated biological material for X-ray microanalysis. J. Micr. Biol. Cell. 22, 215-226.

Echlin P. (1978). Low temperature scanning electron microscopy: a review. J. Microsc. 112, 47-61.

Echlin P. (1979). Low temperature scanning electron microscopy and X-ray microanalysis of biological material. Mikroskopie, 35, 45-49.

Echlin P, Taylor SE. (1986). The preparation and X-ray microanalysis of bulk frozen hydrated vacuolate plant tissue. J. Microsc. 141, 329-348.

Echlin P, Lai CE, Hayes TL. (1982). Low temperature X-ray microanalysis of the differentiating vascular tissue in root tips of Lemna minor L. J. Microsc. 126, 285-306.

Gupta BL, Hall TA. (1981). The X-ray microanalysis of frozen-hydrated sections in scanning electron microscopy: an evaluation. Tissue and Cell, 13, 623-643.

Gupta BL, Hall TA. (1982). Electron probe X-ray microanalysis. Techniques in Cellular Physiology, Part II. Baker PF. (ed.) Elsevier; North Holland, Amsterdam, P128; 1-52.

Gupta BL, Hall TA. (1984). X-ray microanalysis of frozen-hydrated cryosections: problems and results. In: Csanády A, Röhlich P, Szabó D. (eds.): EUREM 1984 Proceedings, Vol. 3. Budapest, pp.: 1665-1674.

Gyenes M, Lustyik Gy, Zs.-Nagy V, Jeney F, Zs.-Nagy I. (1984). Age-dependent decrease of the passive Rb^+ and K^+ permeability of the nerve cell membranes in rat brain cortex as revealed by in vivo measurement of the Rb^+ discrimination ratio. Arch. Gerontol. Geriatr. 3, 11-31.

Hagler HK, Burton KP, Greico CA, Lopez LE, Buja LM. (1980). Techniques for cryo-sectioning and X-ray microanalysis in the study of normal and injured myocardium. Scanning Electron Microsc. 1980; II: 493-498.

Hall TA. (1985). Properties of frozen sections relevant to quantitative microanalysis. J. Microscopy, 141, 319-328.

Hall TA. (1986). The history and the current status of biological electron-probe X-ray microanalysis. Micron and Microsc. Acta, 17, 91-100.

Hall TA, Clarke-Anderson H, Appleton T. (1973). The use of thin specimens for X-ray microanalysis in biology. J. Microsc. 99, 177-182.

Hall TA, Echlin P, Kaufman R. (eds.) (1974). Microprobe Analysis as Applied to Cells and Tissues. Academic Press, New York, London.

Hall TA, Gupta BL. (1983). The localization and assay of chemical elements by microprobe methods. Quart. Rev. Biophys., 16, 279-339.

Hall TA. Gupta BL. (1984). The application of EDXS to the biological sciences. J. Microsc., 136, 193-208.

Hall TA. Gupta BL. (1986). EDS quantification and application to Biology. In Joy DC, Romig Jr. AD, Goldstein JI. (eds.): Principles of Analytical Electron Microscopy, Plenum Press, New York. pp: 219-248

Harvey DMR. (1982). Freeze substitution. J. Microsc. 127, 209-221.

Hodgman ChD, Weast RC, Selby SM. (1959-60). Handbook of Chemistry and Physics, 41st edn, Chemical Rubber Publishing Co. Cleveland, OH.

Ingram FD, Ingram MJ. (1980). Freeze-dried, plastic embedded tissue preparation: A review. Scanning Electron Microsc. 1980; IV: 147-160.

Lustyik Gy. (1986). Age-dependent alterations of the intracellular water and electrolyte content of heart and muscle cells. Arch. Gerontol. Geriatr. 5, 291-296.

Lustyik Gy, Zs.-Nagy I. (1981). Computer assisted method for measuring intracellular water content by means of energy dispersive X-ray microanalysis. EDAX EDITOR, 11, 1-4.

Lustyik Gy, Zs.-Nagy I. (1985). Alterations of the intracellular water and ion concentrations in brain and liver cells during aging as revealed by energy dispersive X-ray microanalysis of bulk specimens. Scanning Electron Microsc. 1985; I: 323-337.

Lustyik Gy, Zs.-Nagy I. (1987). Age-dependent dehydration of postmitotic cells as measured by X-ray microanalysis of bulk specimens. Scanning Microsc. (in press)

Marshall AT. (1980). Quantitative X-ray microanalysis of frozen-hydrated bulk biological specimens. Scanning Electron Microsc. 1980; II: 335-348.

Meyer R, Schmitz M, Zierold K. (1985). The influence of different cryopreparations on the distribution of ions in bullfrog myocard cells. Scanning Electron Microsc. 1985; I: 419-431.

Millner R, Cobet U. (1972). Quantitative electron beam analysis of thick biological tissue samples. Phys. Med. Biol. 17, 736.

Millner R, Cobet U. (1973). Die Elektronenstrahlmikroanalyse zur Untersuchung biologischer Preparate. Wiss. Z. Univ. Halle, 22, 127-130.

Moreton RB. (1981). Electron-probe X-ray microanalysis: Techniques and recent applications in biology. Biol. Rev. 56, 409-461.

Moreton RB, Echlin P, Gupta BL, Hall TA, Weis-Fogh T. (1974). Preparation of frozen-hydrated tissue sections for X-ray microanalysis in the scanning microscope. Nature, 247, 113-116.

Nagy K, Zs.-Nagy I. (1984). Alterations in the molecular weight distribution of proteins in rat brain synaptosomes during aging and centrophenoxine treatment. Mech. Ageing Dev. 28, 171-176.

Newbury DE. (1979). Microanalysis in the scanning electron microscope: progress and prospectives. Scanning Electron Microsc. 1979; II: 1-20.

Panessa BJ. (1974). X-ray microanalysis of biological samples: considerations and problems. J. Submicr. Cytol. 6, 81-94.

Pieri C, Zs.-Nagy I, Zs.-Nagy V, Giuli C, Bertoni-Freddari C. (1977). Energy dispersive X-ray microanalysis of the electrolytes in biological bulk specimen. II. Age-dependent alterations in the monovalent ion contents of cell nucleus and cytoplasm in rat liver and brain cells. J. Ultrastruct. Res. 59, 320-331.

Pieri C, Giuli C, Bertoni-Freddari C, Bernardini A. (1986). Vitamin E deficiency alters the in vivo Rb^+ discrimination of rat brain cortical cells. Arch. Gerontol. Geriatr. 5, 21-31.

Richardson A, Semsei I. (1987). Effect of aging on translation and transcription. in: Rothstein M, (ed.): Review of Biological Research in Aging. Vol.3, Alan R. Liss Inc. New York, pp.: 443-459.

Richardson A, Roberts MS, Rutherford MS. (1985). Aging and gene expression. in: Rothstein M, (ed.): Review of Biological Research in Aging, Alan R. Liss, Inc. Vol 2, New York, pp. 395-419.

Roomans GM. (1979). Standards for X-ray microanalysis of biological specimens. Scanning Electron Microsc. 1979; II: 649-657.

Roomans GM. (1980). Problems in quantitative X-ray microanalysis of biological specimens. Scanning Electron Microsc. 1980; II: 309-320.

Roomans GM. (1981). Quantitative electron probe X-ray microanalysis of biological bulk specimens. Scanning Electron Microsc. 1981; II: 345-356.

Roomans GM, Wei X, Sevéus L. (1982). Cryoultramicrotomy as a preparative method for X-ray microanalysis in pathology. Ultrastruct. Pathol. 3, 65-84.

Russ JC. (1974). X-ray microanalysis in the biological sciences. J. Submicrosc. Cytol. 6, 55-78.

Saubermann AJ, Echlin P, Peters PD, Beeuwkes III. R. (1981a). Application of scanning electron microscopy to X-ray analysis of frozen-hydrated sections. I. Specimen handling techniques. J. Cell Biol. 88, 257-267.

Saubermann AJ, Beeuwkes III. R, Peters PD. (1981b). Application of scanning electron microscopy to X-ray analysis of frozen-hydrated sections. II. Analysis of standard solutions and artificial electrolyte gradients. J. Cell Biol. 88, 268-273.

Semsei I, Szeszák F, Zs.-Nagy I. (1982). In vivo studies on the age-dependent decrease of the rates of total and mRNA synthesis in the brain cortex of rats. Arch. Gerontol. Geriatr. 1, 29-42.

Sitte H. (1982). Instrumentation for cryosectioning. In: 10th Congress on Electron Microscopy, 1982, Vol. I. pp.: 9-18, ed. and published by Deutsche Gesellschaft für Elektronenmikroskopie, Frankfurt AM, 1982.

Somlyó AV, Shuman H, Somlyó AP. (1977). Elemental distribution in striated muscle and the effects of hypertonicity. Electron probe analysis of cryosections. J. Cell Biol. 74, 828-857.

Somlyó AP, Somlyó AV, Shuman H, Stewart M. (1979a). Electron probe analysis of muscle and X-ray mapping of biological specimens with a field emission gun. Scanning Electron Microsc. 1979; II: 711-722.

Somlyó AP, Somlyó AV, Shuman H. (1979b). Electron probe analysis of vascular smooth muscle: composition of mitochondria, nuclei and cytoplasm. J. Cell Biol. 81, 316-335.

Somlyó AP, Bond M, Somlyó AV. (1985). Calcium content of mitochondria and endoplasmic reticulum in liver frozen rapidly in vivo. Nature, Lond. 314, 622-625.

Sumner AT. (1983). X-ray microanalysis: A histochemical tool for elemental analysis. Histochem. J. 15, 501-541.

Umrath W. (1983): Berechnung von Gefriertrocknungszeiten für die elektronenmikroskopische Praparation. Mikroskopie, 40, 9-17.

Von Zglinicki T, Lustyik Gy. (1986). Loss of water from heart muscle cells during aging of rats as measured by X-ray microanalysis. Arch. Gerontol. Geriatr. 5, 283-289.

Wendt-Gallitelli MF, Wolburg H. (1981). Electron probe microanalysis of frozen dried sections of heart muscle prepared using low temperature techniques. Scanning Electron Microsc. 1981; II: 455-462.

Wroblewski J, Müller RM, Wroblewski R, Roomans GM. (1983). Quantitative X-ray microanalysis of semi-thick cryosections. Histochemistry, 77, 447-463.

Zierold K. (1982a). Cryopreparation of mammalian tissue for X-ray microanalysis in STEM. J. Microsc. 125, 149-156.

Zierold K. (1982b). Preparation of biological cryosections for analytical electron microscopy. Ultramicrosc. 10, 45-53.

Zierold K. (1983). X-ray microanalysis of frozen-hydrated specimens. Scanning Electron Microsc. 1983; II: 809-826.

Zierold K. (1986a). Preparation of cryosections for biological microanalysis. In: Müller M, Becker RP, Boyde A, Wolosewick JJ. (eds.): The Science of Biological Specimen Preparation 1985, Scanning Electron Microscopy Inc, AMF O'Hare, Chicago, Illinois, pp.: 119-127.

Zierold K. (1986b). The determination of wet weight concentrations of elements in freeze-dried cryosections from biological cells. Scanning Electron Microsc. 1986; II: 713-724.

Zs.-Nagy I. (1978). A membrane hypothesis of aging. J. Theor. Biol. 75, 189-195.

Zs.-Nagy I. (1979). The role of membrane structure and function in cellular aging: a review. Mech. Ageing Dev. 9, 237-246.

Zs.-Nagy I. (1983). Energy dispersive X-ray microanalysis of biological bulk specimens: a review on the method and its application to experimental gerontology and cancer research. Scanning Electron Microsc. 1983; III: 1255-1268.

Zs.-Nagy I. (1985). Aging of the cellular membrane: Basic principles and pharmacological

interventions. Geriátrika, Vol.I. No.8, 102-111.

Zs.-Nagy I. (1986). Common mechanisms of cellular aging in brain and liver in the light of the membrane hypothesis of aging. In: Kitani K, (ed.): Liver and Aging - 1986, Liver and Brain, Elsevier Science Publishers, Amsterdam, New York, Oxford.pp.: 373-387.

Zs.-Nagy I. (1987a). An attempt to answer the questions of theoretical gerontology on the basis of the membrane hypothesis of aging. In: Aloj Totaro E, Pisanti FA, Glees P, (eds.): Advances in the Biosciences, Vol 64, Pergamon Press, London, Oxford, pp: 393-413.

Zs.-Nagy I. (1987b). Functional consequences of free radical damage to cell membrane. In: Miquel J, Weber H, Quintanilha A, (eds.): CRC Handbook of Biomedicine of Free Radicals and Antioxidants, CRC Press, Inc. Boca Raton, Florida, (in press).

Zs.-Nagy I, Pieri C. (1976). Choice of standards for quantitative X-ray microanalysis of biological bulk specimens. EDAX EDITOR, 6, 28-30.

Zs.-Nagy I, Semsei I. (1984). Centrophenoxine increases the rates of total and mRNA synthesis in the brain cortex of old rats: an explanation of its action in terms of the membrane hypothesis of aging. Exp. Gerontol. 19, 171-178.

Zs.-Nagy I, Pieri C, Giuli C, Bertoni-Freddari C, Zs.-Nagy V. (1977). Energy dispersive X-ray microanalysis of the electrolytes in biological bulk specimen. I. Specimen preparation, beam penetration and quantitative analysis. J. Ultrastruct. Res. 58, 22-33.

Zs.-Nagy I, Nagy K, Zs.-Nagy V, Kalmár A, Nagy É. (1981a). Alterations in total content and solubility characteristics of proteins in rat brain and liver during aging and centrophenoxine treatment. Exp. Gerontol. 16, 229-240.

Zs.-Nagy I, Lustyik Gy, Zs.-Nagy V, Zarándi B, Bertoni-Freddari C. (1981b). Intracellular $Na^+:K^+$ ratios in human cancer cells as revealed by energy dispersive X-ray microanalysis. J. Cell Biol. 90, 769-777.

Zs.-Nagy I, Lustyik Gy, Bertoni-Freddari C. (1982). Intracellular water and dry mass content as measured in bulk specimens by energy-dispersive X-ray microanalysis. Tissue and Cell, 14, 47-60.

Zs.-Nagy I, Lustyik Gy, Lukács G, Zs.-Nagy V, Balázs Gy. (1983). Correlation of malignancy with the intracellular $Na^+:K^+$ ratio in human thyroid tumors. Cancer Res. 43, 5395-5402.

Zs.-Nagy I, Tóth S, Lustyik Gy. (1985). Verification of the membrane hypothesis of aging on the identified giant neurons of the snail Lymnaea stagnalis L. (Gastropoda, Pulmonata) by a combined application of intracellular electrophysiology and X-ray microanalysis. Arch. Gerontol. Geriatr. 4, 53-66.

Discussion with Reviewers

G.M. Roomans: Much of what the author says for the FFFD specimens about ease of preparation etc., applies to thick (Wroblewski et al. 1978) and semi-thick (Wroblewski et al. 1983) cryosections as well. An advantage of these latter specimens may be the greater possibility to combine the analytical work with histochemical studies on adjacent sections. A possible disadvantage would be the relatively high temperature needed for sectioning (-20 to -35°C). Could you comment on this?

Author: In principle any section thicker than the penetration depth of the given accelerating voltage used should be considered as a bulk specimen. Therefore, I am sure that all aspects of our quantitative analysis are valid also for those sections. I am glad to know that the advantages of using bulk specimens have been recognized by others, too, As regards the use of adjacent sections for various histochemical reactions, this is certainly an advantage of the section technique over the FFFD method. The relatively high temperature at cutting sections most likely causes some redistribution of the elements, nevertheless, special investigations may reveal whether this is compromising the results of the given method or not.

G.M. Roomans: By fracturing the specimen you expose an uncontaminated surface, but you also use the middle of the block where freezing is slowest and ice crystals are large, regardless of the freezing method. Do you see any signs of ice crystal damage?

Author: Of course, there is some ice-crystal damage in the FFFD specimens appearing in the form of a certain segregation pattern subsequent to freeze-drying. Cytoplasm and nucleus can be distinguished morphologically because this pattern is different, due to the higher water content of the nuclei as compared to the cytoplasm. However, this is not significant from an analytical point of view, since the total excited volume is much larger than the average "hole"-size of the segregation pattern we observe. As far as I know, even the best and most sophisticated quench-freezing methods can avoid the ice-crystal damage only in a very narrow superficial layer of the block, therefore, it is not possible to look for such zones in the FFFD specimens.

K. Zierold: Your working conditions for X-ray microanalysis are an accelerating voltage of 10-20 kV and a beam current of 1-2 pA for 40 s. This electron dose seems to me very low in order to get a reliable X-ray spectrum. Does this not result in a large scattering of the measured data?

Author: No, because the higher excited volume gives sufficient X-ray counts (400-500 cps) even at such a low beam current. Usual scanning electron microscope values obtainable for intracellular potassium content remain within ± 1 relative %.

Additional Reference

Wroblewski R, Roomans GM, Jansson E, Edström E. (1978). Electron probe X-ray microanalysis of human muscle biopsies. Histochemistry, 55, 281-292.